CHEMICAL NATURE AND BIOLOGICAL SPECIFICITY OF THE SUBSTANCE INDUCING TRANSFORMATION OF PNEUMOCOCCAL TYPES

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Read April 12, 1946, at the Annual Meeting of the American Chemical Society, Symposium on Biochemical and Biophysical Studies on Viruses, Atlantic City, N. J.

The phenomenon of transformation of pneumococcal types requires a brief description in order to provide a background for a discussion of the properties of the transforming substance. The pertinent facts are summarized

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diagramatically in the first slide. The type specificity of pneumococci is dependent upon the presence of a polysaccharide capsule, and is determined by the fact that the polysaccharides are chemically and immunologically distinct in the case of each of the many types of this organism. By growth in specific antisera, non-encapsulated variants can be derived from encapsulated strains. The non-encapsulated variants, designated as R forms, are avirulent for laboratory animals and lacking in type specificity. In the technique employed at present, transformation of type is accomplished by growing such non-encapsulated cells in a special serum broth to which has been added material extracted from encapsulated pneumococci of a heterologous type. The production of a new polysaccharide capsule is induced in the R cells so that they acquire the type-specificity of the organism from which the extract was obtained. The transfor-

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mation is illustrated by a photograph of colonies of an R strain derived from Type II and colonies of the same strain after induction of transformation by growth in the presence of a transforming extract from Type III pneumococci. The

change so induced is permanent, and furthermore the transformed cells can themselves serve as a source of the specific transforming substance. Thus, the transforming substance induces a predictable and heritable modification and is itself reproduced in the transformed cells.

There are certain striking analogies between the biological properties of the agent that induces transformation and those of viruses and genes. For example, as in the case of viruses the transforming agent acts only on susceptible living cells; it is transmissible in series and can subsequently be recovered in amounts far in excess of that originally used as inoculum. As in the case of genes, the transforming substance behaves as a heritable unit in that it induces predictable and durable alterations in cellular structure and function and is itself reduplicated in daughter cells through successive generations. It intervenes in the metabolism of the R cell giving rise to the synthesis of a new capsular substance, which in turn endows the cells with distinctive and biologically specific characters not possessed by the parent strain. Although reasoning by analogy is more often fallacious than valid, these similarities in biological behavior, together with the accumulated evidence on the chemical nature of the transforming substance, may serve as justification for including this topic in a symposium on the "Biochemical and Biophysical Studies on Viruses."

When it was found that crude extracts of the bacterial cells possessed these remarkable biological properties, it became of interest to determine the chemical nature of the substance responsible for their activity. Efforts in this direction led to the isolation of the active transforming substance from Pneumococcus Type III in the form of a viscous desoxyribonucleic acid fraction. In view of the apparent chemical uniformity of the nucleic acids even though isolated from diverse sources, it seemed doubtful at first that the biological activity of the pneumococcal extract could be attributed to the desoxyribonucleic acid contained therein. However, several different lines of analysis provided a consistent body of evidence which indicated that the purified transforming substance is composed almost exclusively of desoxyribonucleic acid and that biological activity is indeed a property of the nucleic acid. For example, the results of elementary chemical analysis of the purified material conformed closely to the values characteristic of nucleic acid, and on the basis of the nitrogen-phosphorus ratio it appeared that little protein could be present as impurity. This finding was further confirmed by the fact that the presence of protein could not be demonstrated by any of several qualitative tests. In addition, serological reactions carried out with high-titer antisera prepared against the homologous type of Pneumococcus were ngative, indicating the absence of serologically

reactive pneumococcal protein and polysaccharides. When studied in the ultracentrifuge and electrophoresis apparatus, the purified material moved with a single boundary, and it was found that biological activity migrated with the optically observed boundary. Further suggestive evidence is provided by the fact that as little as 0.003 μg. of the purified desoxyribonucleic acid fractions suffices to induce transformation.

Because of the possibility that some other substance, present in minute amounts and intimately associated with the nucleic acid fraction, might be responsible for activity, further evidence was sought by the use of enzymatic techniques. It was shown that while crystalline trypsin, chymotrypsin and ribonuclease are without effect, the activity of the transforming substance is rapidly and completely destroyed by certain crude enzyme preparations, and that these same enzyme preparations also possess the ability to hydrolyze highly polymerized desoxyribonucleic acid from animal sources. To provide a more specific reagent for application to this problem, the isolation and purification of the enzyme which hydrolyzes desoxyribonucleic acid was undertaken.

This enzyme, termed desoxyribonuclease, has been isolated in a highly active form by salt fractionation of an acid extract of beef pancreas. This is the same type of acid extract of pancreas from which Northrop and Kunitz

obtained crystalline trypsin, chymotrypsin and ribonuclease. The desoxyribonuclease is precipitated from the acid extract by relatively low concentrations of ammonium sulfate and is thus readily separated from the bulk of the other enzymes present. Purified preparations of desoxyribonuclease have no action on ribonucleic acid and contain only minute traces of proteolytic enzymes. The enzyme has been found to require activation by magnesium ion and it displays little or no catalytic property in the absence of the metallic activator.

Throughout the procedures of isolation and purification, quantitative estimation of desoxyribonuclease activity was accomplished by measuring the effect on the viscosity of solutions of calf-thymus desoxyribonucleate. Since the final end-products of the reaction are soluble in mineral acid, activity can also be measured by the decrease in the acid precipitability of the nucleate.

3 The activity of purified desoxyribonuclease is illustrated in the third slide, in which is presented the results of viscosimetric determinations of the activity of three separate preparations at the same final enzyme concentration, 0.02 μg. per cc. The determinations were made at 30°C. with a 0.1 percent solution of sodium desoxyribonucleate from calf-thymus in veronal buffer at pH 7.5 and in the presence of 0.003 M $MgSO_4$. Under these conditions, a measurable effect is obtained at enzyme concentrations below 0.01 μg. per cc.

The purified desoxyribonuclease, which was thus shown to be highly active and specific in hydrolyzing animal nucleic acid, was tested for its effect on the transforming activity of the purified pneumococcal nucleate. In this type of experiment the same conditions with respect to temperature, pH and magnesium ion concentration were used as in the case of the viscosimetric determinations using animal nucleate. Samples of the purified transforming substance from Pneumococcus Type III were mixed with varying concentrations of desoxyribonuclease for 30 minutes, and the reaction was stopped by heating at 60°. for 15 minutes. Serial tenfold dilutions were then prepared from the reaction mixtures and each dilution was tested for transforming activity in quadruplicate tubes. All tubes were inoculated with a susceptible strain of R pneumococcus and incubated at 37° for 18 to 24 hours. The occurrence of transformed type III cells was determined by bacteriological and serological tests. (SLIDE 4). In the table, "R only" indicates that no transformation has occurred, and S III denotes the recovery of Type III organisms. It will be seen that after treatment with the enzyme at final concentrations of 0.01 and 0.005 μg. per cc., the transforming substance is completely inactivated, and that only a slight amount of residual activity remains after treatment with 0.0025 μg. per cc. Even at the lowest enzyme concentration there is a definite

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effect on activity as compared with that of untreated control material.

The data from the enzymatic studies have therefore provided strong supporting evidence for the view that the biological activity of the transforming substance is a property of the desoxyribonucleic acid. Certainly there can be little doubt that desoxyribonucleic acid must be present in its intact, highly polymerized form. The accumulated evidence makes it extremely unlikely that small traces of some other specific substance, such as a protein, could be responsible for the manifestation of transforming activity.

The ease with which the transforming substance can be inactivated by enzymatic action stands in direct contrast to most of the accumulated experience with animal and plant viruses and bacteriophage, which have been shown to be highly resistant to inactivation by nucleases, and in most instances by other enzymes as well. It may be that this is an indication of a fundamental chemical difference between the transforming substance and the viruses, a difference that has already been suggested by the apparent absence of protein and of serological activity.

The initial studies on the isolation and properties of the transforming substance were carried out with Pneumococcus Type III. Recently, purified material has been obtained from other types, specifically Type II and Type VI.

In the case of these other types, biological activity is again associated with a desoxyribonucleic acid fraction. The isolation of the active material from all three types is greatly facilitated by the use of a new method which is based on information concerning the properties of the enzyme, desoxyribonuclease. Pneumococcal cells contain desoxyribonuclease, so that lysis of living cells is accompanied by enzymatic breakdown of desoxyribonucleic acid and consequent destruction of the transforming substance. However, since desoxyribonuclease is magnesium activated, effective inhibition can be achieved by the use of citrate which serves to remove free magnesium ion from the system; consequently lysis of living pneumococcal cells in the presence of citrate releases the active material without coincident destruction. Use of this technique has resulted in fivefold greater yields of the active desoxyribonucleic acid.

It is of interest that preparations of purified material from two separate types - for example, Type II and Type III - cannot be distinguished from one another by chemical, physical or even serological means, and that only their specificity in the biological test of transforming activity serves to differentiate them. Although differences in chemical structure or configuration must be assumed to underlie the differences in biological specificity, no other means for their detection are available at present.

There are indications that the problem of the chemical basis for the specificity of nucleic acids may prove to be extremely complex. A desoxyribonucleic acid fraction can be obtained from unencapsulated pneumococci in a yield comparable to that of the same fraction from Type III cells. The nucleic acid of the unencapsulated cells can be differentiated from Type III transforming substance only by the fact that it is wholly devoid of transforming activity. It is possible that the nucleic acid of the R pneumococcus is concerned with innumerable other functions of the bacterial cell in a way similar to that in which capsular development is controlled by the transforming substance. The desoxyribonucleic acid from Type III pneumococci would then necessarily comprise not only molecules endowed with transforming activity, but in addition a variety of others which determine the structural and metabolic activities possessed in common by both the encapsulated and unencapsulated forms. If these considerations have any foundation in fact, the task of discovering the chemical basis of the biological specificity of desoxyribonucleic acids is complicated by the fact that any given preparation will represent a large number of entities of diverse specificity.

An example of an analogous situation is provided by the gamma globulin fraction of mammalian sera. When properly prepared, gamma globulin appears to

be chemically and physically homogeneous, although it can be shown to contain a number of antibodies of varying specificity, as well as numerous enzymes. Without the corresponding specific antigens or substrates these diverse specificities cannot be detected. In the case of nucleic acid, the only means at present available for demonstrating specificity are the techniques employed in transformation of pneumococcal types.

The mechanism of the change brought about by the transforming substance is not understood at present. One supposes that the specific desoxyribonucleic acid enters a susceptible cell and sets in progress a chain of biochemical events which culminate in the synthesis of the capsular polysaccharide. According to this view, the nucleic acid causes the formation of a new enzyme or enzyme system which in turn elaborates a secondary product - the specific polysaccharide.

An experimental approach to at least one phase of the mechanism of transformation is provided by a study of the rôle of serum in the transforming system. Unknown substances present in certain sera and serous fluids are required for the induction of transformation <u>in vitro</u> by the specific desoxyribonucleic acid. The contribution of serum is non-specific, since the same serum can be used in a variety of different transformations. Some understanding of the nature of these non-specific accessory factors is essential to

clarification of the mechanism of pneumococcal transformation, and is probably important in any projected extension of the techniques of transformation to cells of higher organisms. Work in progress gives indications that the serum factor is complex and consists of three separate components. Although no final statement can be made concerning the identity of these components, it would appear that their function, in part at least, is concerned with the alteration of the surface of the bacterial cell so that the specific desoxyribonucleic acid is taken up or adsorbed.

It will be observed from the foregoing discussion that while the pneumococcal transforming substance is virus-like in certain of its properties, there is some evidence inconsistent with its classification with the viruses, despite the diversity of this group of agents. However, if one accepts the validity of the view that the biological specificity of the transforming substance is the property of a desoxyribonucleic acid, the results of the present study serve to focus attention on the nucleic acid component of virus nucleoproteins. In addition to its probable rôle in the self-reproduction of the virus molecule, the nucleic acid moiety may carry a specificity which is a determining factor in the ultimate structure of the virus.